Environmental Microbiology (2007) **9**(2), 445–452 doi:10.1111/j.1462-2920.2006.01161.x

The bacterial storage compound poly-β-hydroxybutyrate protects *Artemia franciscana* from pathogenic *Vibrio campbellii*

Tom Defoirdt,[1,2] Dirk Halet,[1] Han Vervaeren,[1] Nico Boon,[1] Tom Van de Wiele,[1] Patrick Sorgeloos,[2] Peter Bossier[2] and Willy Verstraete[1]*

[1]*Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, 9000 Gent, Belgium.*

[2]*Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Rozier 44, 9000 Gent, Belgium.*

Summary

Infections caused by antibiotic-resistant luminescent *Vibrio*s can cause dramatic losses in aquaculture. In this study, the short-chain fatty acid β-hydroxybutyrate and its polymer poly-β-hydroxybutyrate were investigated as possible new biocontrol agents. β-Hydroxybutyrate was shown to completely inhibit the growth of pathogenic *Vibrio campbelli* at 100 mM. Moreover, the addition of 100 mM of this fatty acid to the culture water of *Artemia* nauplii infected with the *V. campbelli* strain significantly increased the survival of the nauplii. As *Artemia* is a non-selective and particle filter feeder, we also investigated whether poly-β-hydroxybutyrate particles could be used to protect *Artemia* from the pathogenic *V. campbellii*. The addition of 100 mg l^{-1} poly-β-hydroxybutyrate or more to the *Artemia* culture water offered a preventive and curative protection from the pathogen as a significantly enhanced survival was noticed. If added as a preventive treatment, a complete protection of infected nauplii (no significant mortality compared with uninfected nauplii) was observed at 1000 mg l^{-1} poly-β-hydroxybutyrate. Our data indicate that the use of poly-β-hydroxybutyrate might constitute an ecologically and economically sustainable alternative strategy to fight infections in aquaculture.

Received 23 March, 2006; accepted 13 September, 2006. *For correspondence. E-mail Willy.Verstraete@UGent.be; Tel. (+32) 9 264 59 76; Fax (+32) 9 264 62 48.

Introduction

Disease outbreaks are considered to be a significant constraint to the development of the aquaculture sector (Subasinghe *et al*., 2001). Infections caused by luminescent *Vibrio*s can cause dramatic losses in the intensive rearing of molluscs, finfish, lobsters and especially shrimp (Pass *et al*., 1987; Alvarez *et al*., 1998; Lavilla-Pitogo *et al*., 1998; Diggles *et al*., 2000; Zhang and Austin, 2000). The frequent use of antibiotics in order to control these bacteria has resulted in the development of (multiple) resistance (Teo *et al*., 2000; 2002; Molina-Aja *et al*., 2002) and consequently, these compounds are ineffective in controlling luminescent vibriosis (Karunasagar *et al*., 1994). Therefore, there is an urgent need for alternative control techniques.

Several studies have shown that short-chain fatty acids inhibit the growth of yeast and enterobacteria such as *Salmonella typhimurium*, *Escherichia coli* and *Shigella flexneri* (Bergeim, 1940; Wolin, 1969; Cherrington *et al*., 1991; Bearson *et al*., 1997; Sun *et al*., 1998; Van Immerseel *et al*., 2003). In fact, short-chain fatty acids are already used in commercial mixtures to control *Salmonella* in poultry (Van Immerseel *et al*., 2002). In the undissociated form, the short-chain fatty acids can pass the cell membrane of bacteria and dissociate in the more alkaline cytoplasm, thereby increasing the intracellular concentration of protons (Kashket, 1987; Cherrington *et al*., 1991). Consequently, the cells have to spend energy in order to maintain the intracellular pH at the optimal level. This energy cannot be used for other metabolic processes and therefore, growth of the cells is inhibited.

A wide variety of microorganisms are known to produce polymers of the fatty acid β-hydroxybutyrate as an intracellular energy and carbon storage compound (Anderson and Dawes, 1990; Madison and Huisman, 1999). Poly-β-hydroxybutyrate (PHB) is deposited intracellularly in the form of inclusion bodies in a fluid, amorphous state (Amor *et al*., 1991). After death and cell lysis, the polymer is released in a partially crystalline state (Doi, 1995). The ability to degrade extracellular PHB depends on the secretion of extracellular PHB depolymerase enzymes and is widely distributed among bacteria and fungi (Jendrossek, 1998; Jendrossek and Handrick, 2002). The

© 2006 The Authors
Journal compilation © 2006 Society for Applied Microbiology and Blackwell Publishing Ltd

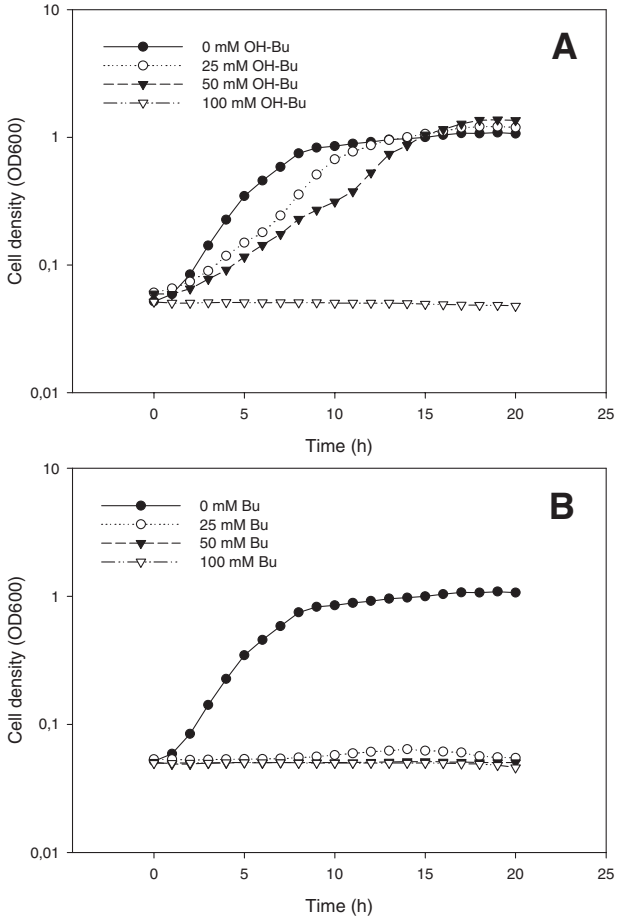

Fig. 1. Growth of *Vibrio campbellii* LMG21363 (OD_{600}) at pH 6 in the presence of β-hydroxybutyrate (A) or butyrate (B). The data points are the mean values of three replicates (error bars are too small to be visible) and are representative of two independent experiments.

extracellular PHB depolymerase of *Comamonas testosteroni* is well characterized; the enzyme was found to hydrolyse PHB into β-hydroxybutyrate monomers (Mukai *et al*., 1993; Kasuya *et al*., 1994; Shinomiya *et al*., 1997). Apart from microbial degradation, PHB has also been shown to be degraded in animal tissues (Saito *et al*., 1991; Gogolewski *et al*., 1993; Freier *et al*., 2002) and to be hydrolysed under acidic and alkaline conditions (Yu *et al*., 2005).

In this study, we investigated whether β-hydroxybutyrate could inhibit the growth of a pathogenic *Vibrio campbellii* strain and whether the addition of this fatty acid to the culture water could improve the survival of infected *Artemia* nauplii. As *Artemia* is a non-selective and particle filter feeder grazing on particles with a diameter preferentially smaller than 50 μm (Sorgeloos *et al*., 1986), we also investigated whether PHB particles (with an average diameter of 30 μm) could be used to deliver β-hydroxybutyrate to the *Artemia* gut and whether this would result in a protection from the pathogenic *V. campbellii*.

Results

The effect of β-hydroxybutyrate on growth of V. campbellii *LMG21363*

The short-chain fatty acid β-hydroxybutyrate was tested for its ability to inhibit the growth of the pathogenic strain *V. campbellii* LMG21363. At pH 6, the growth of the strain was completely inhibited if the medium was supplemented with 100 mM β-hydroxybutyrate (Fig. 1A). The growth of *V. campbellii* was not completely inhibited in the presence of lower concentrations of the fatty acid. However, the growth rate was clearly lower than for the control and inversely related to the concentration of β-hydroxybutyrate.

Butyrate was used as a reference fatty acid because its growth-inhibitory effect is well documented and because it has approximately the same pKa value as β-hydroxybutyrate. The inhibition of growth by butyrate was more pronounced than by β-hydroxybutyrate. At pH 6, the growth of the pathogen was completely inhibited even at the lowest concentration (25 mM; Fig. 1B).

The growth-inhibitory effect of both fatty acids was clearly pH-dependent because at pH 7, growth of the pathogen was only inhibited in medium supplemented with the highest concentration of butyrate and at pH 8, no inhibition was observed (data not shown).

The effect of β-hydroxybutyrate on the survival of Artemia *nauplii infected with* V. campbellii *LMG21363*

In a first *in vivo* challenge test, the effect of β-hydroxybutyrate on the survival of *Artemia* nauplii infected with the pathogenic strain *V. campbellii* LMG21363 was investigated. The fatty acid significantly enhanced the survival of the infected nauplii when added at a concentration of 100 mM (Table 1). The survival of the nauplii with 25 mM β-hydroxybutyrate was also higher

Table 1. Percentage survival of *Artemia* nauplii (mean ± standard error of three replicates) after 2 days challenge with *Vibrio campbellii* LMG21363.

Treatment	Survival (%)
Control	80 ± 3
LMG21363	12 ± 2
LMG21363 + β-hydroxybutyrate (25 mM)	38 ± 15
LMG21363 + β-hydroxybutyrate (100 mM)	40 ± 3[a]
LMG21363 + butyrate (25 mM)	48 ± 2[a]
LMG21363 + butyrate (100 mM)	50 ± 5[a]

a. Significant difference in survival with infected nauplii without the addition of fatty acid ($P < 0.01$).
β-Hydroxybutyrate or butyrate were added at 25 or 100 mM to the *Artemia* culture water at the start of the experiment.

© 2006 The Authors
Journal compilation © 2006 Society for Applied Microbiology and Blackwell Publishing Ltd,

than the survival of infected nauplii without fatty acid. However, the difference was not significant because of a high variation. Butyrate, which was again used as a reference, significantly enhanced the survival of the infected nauplii for both concentrations tested (Table 1). Both fatty acids had no effect on the survival of uninfected nauplii (data not shown).

The effect of PHB particles on the survival of starved Artemia *nauplii*

A starvation test was performed in order to verify whether *Artemia* could obtain energy from PHB particles, which would indicate that the particles are degraded in the intestinal tract of the nauplii. The addition of PHB to the culture water resulted in a prolonged survival of the nauplii. Two different feeding regimes were applied: no feed or an autoclaved suspension of LVS3 bacteria. In the treatments without LVS3, there was a significant difference in survival between the treatment with and the one without PHB after 1, 2 and 3 days of starvation (Fig. 2A). The nauplii fed with LVS3 survived longer than unfed nauplii. In this case, there was a significant difference in survival between the treatment with and the one without PHB after 2, 3 and 4 days of starvation (Fig. 2B). This indicates that the nauplii can obtain energy from the PHB particles and therefore, the PHB must (at least partially) be degraded in the gut.

Microscopic analysis of LVS3-fed nauplii after 2 days of starvation showed that the gut of nauplii in the treatment without PHB was empty (Fig. 3A), whereas in the treatment with PHB, the gut was almost completely filled (Fig. 3B). Epifluorescence images of Nile Blue A-stained nauplii showed that the gut content of PHB-treated nauplii was brightly fluorescent (Fig. 3D) and could clearly be distinguished from the (auto)fluorescence of the nauplii (Fig. 3C) which indicates that the gut content had a high PHB concentration.

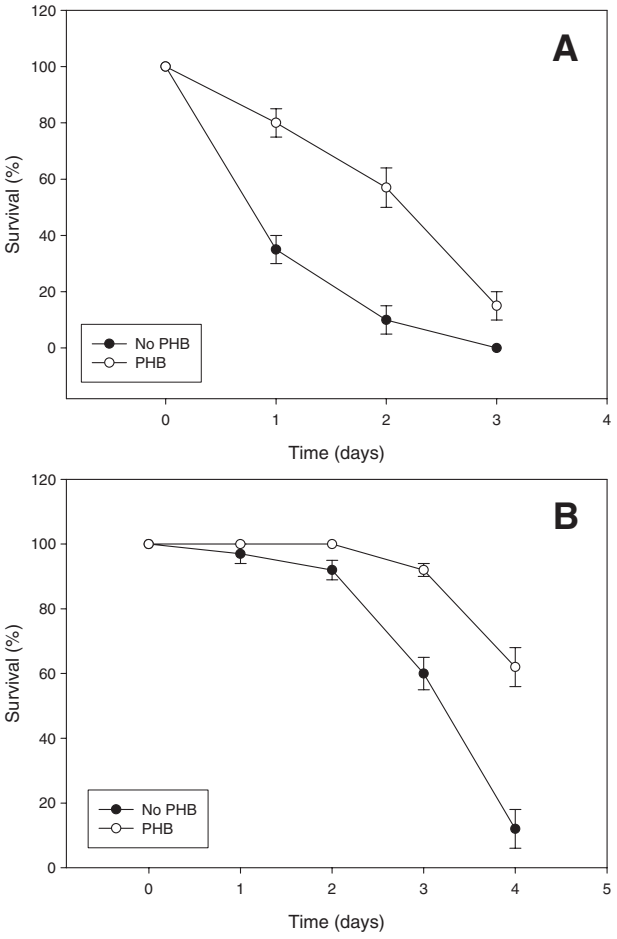

Fig. 2. Survival of starved *Artemia* nauplii (mean ± standard error of three replicates) with 1000 mg l^{-1} PHB and without PHB. The nauplii were either not fed (A) or fed with autoclaved LVS3 bacteria at the start of the experiment (B).

Bacterial depolymerization of PHB particles

The results of the *in vivo* starvation test indicate that PHB particles are (at least partially) degraded in the gut of *Artemia* nauplii. However, enhancing the PHB degradation might still improve the protection from the virulent *V. campbellii* isolate. In order to investigate the effect of enhanced depolymerization of PHB, we decided to apply extracellular PHB depolymerase producing bacteria. As *C. testosteroni* has been reported before to be able to produce this enzyme, different *C. testosteroni* strains were screened for PHB depolymerization by streaking them onto agar containing PHB particles as the sole C-source and checking for the formation of a clearing zone. *Comamonas testosteroni* LMG19554 showed excellent PHB depolymerase activity as a clearing zone was present around the colonies already after 1 day of incubation. After 2 days of incubation, a large clearing zone with complete clearing of the medium was observed (Fig. 4). Extracellular PHB depolymerase production was also assessed for *V. campbellii* LMG21363. However, no clearing of the medium could be observed (data not shown).

The effect of PHB particles on the survival of Artemia *nauplii infected with* V. campbellii *LMG21363*

A last *in vivo* experiment aimed at testing whether the addition of PHB particles would result in a protection from the pathogenic *V. campbellii*. The survival of infected *Artemia* nauplii was found to be proportional to the concentration of PHB added to the culture water (Table 2).

The addition of PHB together with the pathogen significantly enhanced the survival of the infected nauplii when

© 2006 The Authors

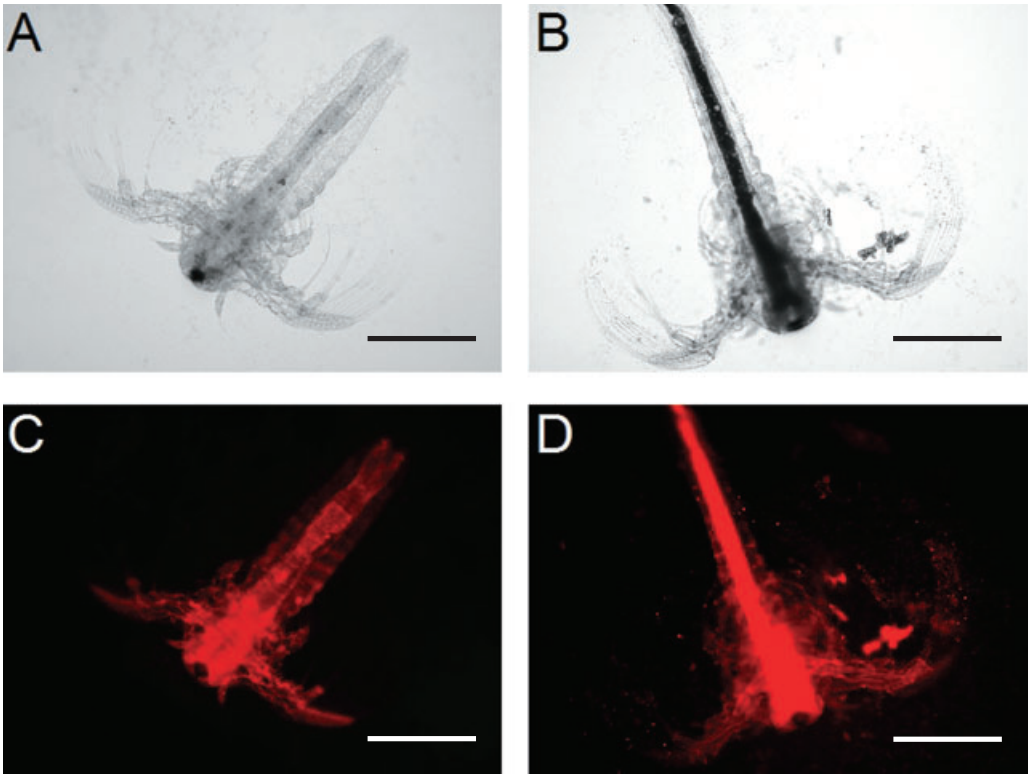

Fig. 3. Light (A and B) and epifluorescence microscopy (C and D) images of Nile Blue A-stained *Artemia* nauplii after 2 days of starvation without (A and C) and with (B and D) PHB, added to the culture water at 1000 mg l^{-1} at the start of the experiment. Bar = 250 μm.

added at 100 mg l^{-1} and 1000 mg l^{-1}. A complete protection (no significant difference in survival with uninfected nauplii) was observed at the highest concentration. The PHB depolymerizing strain *C. testosteroni* LMG19554 was added in order to study the effect of an enhanced PHB depolymerization and the strain significantly improved the performance of the PHB at 100 mg l^{-1}. Importantly, it had no effect on the survival of infected nauplii in the absence of PHB.

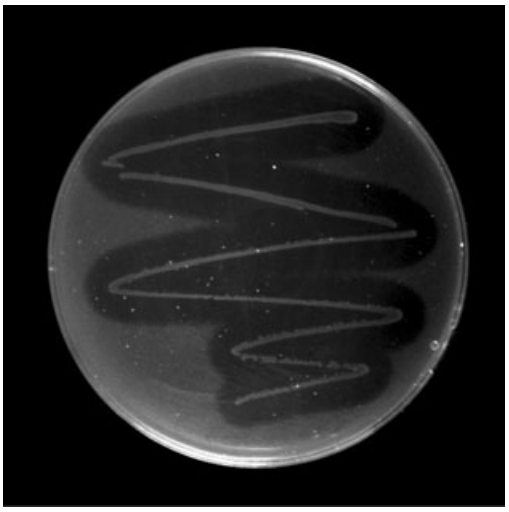

Fig. 4. Poly-β-hydroxybutyrate depolymerization by *Comamonas testosteroni* LMG19554 after 2 days of incubation at 28°C. Clearing of the medium around the growing strain indicates the hydrolysis of PHB into water-soluble products by extracellular PHB depolymerase.

If the PHB particles were added 1 day after the addition of the pathogen, a similar but less pronounced effect was noticed (Table 2). The addition of PHB significantly enhanced the survival of the infected nauplii when added at 100 mg l^{-1} and 1000 mg l^{-1} as was the case if the particles were added together with *V. campbellii* LMG21363. However, there was still significant mortality in infected *Artemia* treated with 1000 mg l^{-1} PHB.

Discussion

In this study, we evaluated the short-chain fatty acid β-hydroxybutyrate and its polymer PHB as possible new biocontrol agents for aquaculture, using *Artemia* infected with a pathogenic *V. campbellii* strain as a model. The strain used in our model system, *V. campbellii* LMG21363, is as far as we know the most virulent *Artemia* pathogen reported thus far. In a first experiment, β-hydroxybutyrate was found to be able to inhibit the growth of the virulent *V. campbellii* strain *in vitro*. The inhibition was clearly pH-dependent because at a pH

© 2006 The Authors

Table 2. Percentage survival of *Artemia* nauplii (mean ± standard error of three replicates) after 2 days challenge with *Vibrio campbellii* LMG21363.

Treatment	Survival (%)	
	Without *Comamonas*	With *Comamonas*
Control	87 ± 3	NT
LMG21363	17 ± 2	18 ± 2
LMG21363 + PHB (10 mg l^{-1}; start)	22 ± 4	20 ± 5
LMG21363 + PHB (100 mg l^{-1}; start)	40 ± 3[a]	62 ± 4[a]
LMG21363 + PHB (1000 mg l^{-1}; start)	90 ± 3[a]	92 ± 3[a]
LMG21363 + PHB (10 mg l^{-1}; day 1)	18 ± 7	NT
LMG21363 + PHB (100 mg l^{-1}; day 1)	38 ± 2[a]	NT
LMG21363 + PHB (1000 mg l^{-1}; day 1)	60 ± 6[a]	NT

a. Significant difference in survival with infected nauplii without the addition of PHB ($P < 0.01$). PHB particles were added to the culture water either at the start of the experiment (with or without the extracellular PHB depolymerase producing strain *Comamonas testosteroni* LMG19554) or after 1 day. NT, not tested.

of 7 or higher, no inhibition was observed. Similar pH-dependent effects have been reported for the inhibition of the growth of *Salmonella enteritidis* and *S. typhimurium* by short-chain fatty acids (McHan and Shotts, 1993; Durant *et al*., 2000; Van Immerseel *et al*., 2003). The pH-dependent effect can be explained by the fact that the fatty acids can pass the cell membrane only in their undissociated form (Sun *et al*., 1998), which will be more dominant at lower pH (according to the Henderson–Hasselbach equation).

As β-hydroxybutyrate and butyrate were found to inhibit the growth of *V. campbellii* LMG21363, a first *in vivo* challenge test aimed at investigating whether both fatty acids could protect *Artemia* nauplii from the pathogen. The addition of 100 mM of the fatty acids to the *Artemia* culture water significantly increased the survival of infected nauplii. Apart from inhibiting the growth of *V. campbellii*, the protection offered by the short-chain fatty acids might partially be explained by a feed effect. Indeed, it has been shown before that *Artemia* can use β-hydroxybutyrate as energy source (Weltzien *et al*., 2000) and that the susceptibility of *Artemia* nauplii to pathogenic bacteria decreases if they are fed a better-quality feed (Marques *et al*., 2005).

The effective fatty acid concentrations are rather high and consequently it would economically not be feasible to dose the fatty acids in the culture water of an aquaculture system in order to protect the animals. Moreover, the addition of high levels of organic carbon in the water would give rise to an excessive growth of heterotrophic bacteria that might have a negative effect on the health of the animals (because of oxygen depletion and/or because the growing bacteria could be pathogenic). Consequently, we searched for a more elegant method to deliver the fatty acids into the gut. *Artemia* is a non-selective and particle filter feeder (Sorgeloos *et al*., 1986) and therefore, we investigated whether PHB particles could be used for this purpose. An *in vivo* challenge test with PHB particles revealed that the survival of *Artemia* nauplii infected with *V. campbellii* LMG21363 was proportional to the PHB concentration in the culture water, with a significantly improved survival at 100 mg l^{-1} and 1000 mg l^{-1}. If added as a preventive treatment, a complete protection (no significant difference in survival with uninfected nauplii) was observed at the highest PHB concentration tested. Interestingly, the addition of PHB to the culture water also showed to have a curative effect as the addition of PHB 1 day after the addition of the pathogen also resulted in a significant protection. However, the protection was less pronounced than if the PHB was added together with the pathogen as there was still significant mortality at the highest concentration tested. As far as we know, this is the first report showing that the bacterial storage compound PHB can be used to control bacterial infections.

We hypothesize that the PHB particles are (partially) degraded into β-hydroxybutyrate in the *Artemia* gut and that the release of this fatty acid protects the nauplii from the pathogen in two ways, i.e. by providing the nauplii from energy and by inhibiting the growth of the pathogen (see earlier). Unfortunately, we were not able to detect β-hydroxybutyrate in the *Artemia* nauplii because of their small dimensions and although the particles were not sterile, no degradation of the polymer could be observed even after prolonged incubation of mineral medium containing the particles as the sole source of carbon. However, the fact that the addition of PHB particles to the culture water resulted in a significantly prolonged survival of starved nauplii confirms the hypothesis of PHB degradation *in vivo*. Indeed, in order to provide the nauplii with energy, the particles must have been (partially) degraded into water-soluble products (i.e. β-hydroxybutyrate monomers and oligomers). In fact, if we assume that accumulation of PHB particles in the gut leaves open only 10% of the gut volume for a liquid phase then only about 0.1% of the PHB needs to be degraded into β-hydroxybutyrate in order to yield a concentration that is growth-inhibitory towards *V. campbellii*. This value is probably an overestimation as the volume left open by the particles was

© 2006 The Authors
Journal compilation © 2006 Society for Applied Microbiology and Blackwell Publishing Ltd,

usually smaller than 10% (see microscopic images). Nevertheless, a degradation of 0.1% is quite possible if compared with what has been found *in vivo* in rat tissues (Gogolewski *et al*., 1993). Moreover, the presence of digestive enzymes has been shown to increase the degradation of PHB about threefold (Freier *et al*., 2002). However, as far as we know, no data have been published yet on the fate of the polymer inside the gut of animals and therefore, further research is needed in order to verify by which mechanism PHB is degraded in the shrimp gut (i.e. enzymatic and/or physico-chemical).

If compared with β-hydroxybutyrate, the addition of PHB particles was about 100 times more efficient. Indeed, only 100 mg l^{-1} PHB was needed to obtain a similar effect as was observed with 100 mM (≈ 10 g l^{-1}) β-hydroxybutyrate (Tables 1 and 2). An even higher effect was obtained by the addition of the PHB depolymerase producing strain *C. testosteroni* LMG19554 together with the PHB particles, which can be explained by an improved release of β-hydroxybutyrate. Importantly, the *C. testosteroni* strain had no effect on the survival of infected *Artemia* in the absence of PHB, excluding any other protective effect. The particles used in this study had an average diameter of 30 μm. However, the release of β-hydroxybutyrate might be more efficient if the PHB particles were smaller (resulting in a higher surface/mass ratio) and therefore, the effect might still be improved by adding smaller particles. We are currently investigating whether it would be possible to use PHB-accumulating bacteria (without extraction of the polymer) for this purpose as the PHB is present in a much smaller size inside these bacteria (< 1 μm; Anderson and Dawes, 1990). The use of these bacteria might constitute an economically interesting biocontrol strategy for aquaculture.

In conclusion, our results indicate that short-chain fatty acids with a growth-inhibitory effect towards pathogenic bacteria might be useful to treat bacterial infections in aquaculture. Furthermore, formulating the short-chain fatty acid β-hydroxybutyrate as a polymer, the well-known bacterial storage compound PHB, resulted in a more effective protection. This indicates that the addition of PHB (or PHB-accumulating bacteria) might constitute an ecologically and economically sustainable alternative anti-infective strategy for aquaculture.

Experimental procedures

Bacterial strains and growth conditions

Vibrio campbellii LMG21363 (= PN9801; Soto-Rodriguez *et al*., 2003) and *C. testosteroni* LMG19554 (= I2; Boon *et al*., 2000) were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium). *Vibrio campbellii* LMG21363 and *Aeromonas hydrophila* LVS3 (Verschuere *et al*., 1999) were grown in Marine Broth (Difco Laboratories, Detroit, USA). *Comamonas testosteroni* LMG19554 was grown in LB medium. All strains were grown at 28°C with shaking (100 rotations per minute).

The effect of β-hydroxybutyrate and butyrate on the growth of V. campbellii *LMG21363*

Vibrio campbellii LMG21363 was grown overnight in LB medium on a shaker at 28°C. Subsequently, the suspension was diluted 1:50 (v/v) in LB medium supplemented with butyrate (Sigma-Aldrich, Bornem, Belgium) or β-hydroxybutyrate (Sigma-Aldrich, Bornem, Belgium). For each compound, three solutions were made with the following concentrations: 25, 50 and 100 mM. LB medium without supplements was used as a control. The pH of all solutions was adjusted to, respectively, 6, 7 or 8. After inoculation, the suspensions were incubated at 28°C in a static mode. Growth was monitored by measuring the optical density (OD_{600}) during 20 h. Each treatment was performed in triplicate.

Extracellular PHB depolymerase assay

Extracellular PHB depolymerase production was assessed in a qualitative way by streaking strains on solid medium containing PHB particles as the sole C-source. The medium used in the experiments contained 500 mg l^{-1} PHB particles (average diameter 30 μm; Goodfellow, Huntingdon, UK), 1 g l^{-1} NH_4Cl, 1 g l^{-1} KNO_3, 5 g l^{-1} artificial seasalt (Aquarium Systems, Sarrebourg, France) and 15 g l^{-1} agar. The plates were incubated for up to 4 days at 28°C and examined daily for the presence of a clearing zone around the colonies.

Axenic hatching of Artemia franciscana

All challenge tests were performed with high-quality hatching cysts of *A. franciscana* (EG® Type, batch 6940, INVE Aquaculture, Baasrode, Belgium). Two hundred milligrams of cysts were hydrated in 18 ml of tap water during 1 h. Sterile cysts and nauplii were obtained via decapsulation, adapted from the protocol described by Marques and colleagues (2004). Briefly, 660 μl of NaOH (32%) and 10 ml of NaOCl (50%) were added to the hydrated cyst suspension. The decapsulation was stopped after 2 min by adding 14 ml of $Na_2S_2O_3$ (10 g l^{-1}). During the reaction, 0.22 μm filtered aeration was provided. The decapsulated cysts were washed with autoclaved artificial seawater containing 35 g l^{-1} of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts were re-suspended in a 50 ml tube containing 30 ml of filtered and autoclaved artificial seawater and hatched for 30 h on a rotor (4 min^{-1}) at 28°C with constant illumination (approximately 2000 lux).

Preparation of the inocula for in vivo *experiments*

Vibrio campbellii LMG21363 was stored in 40% glycerol at –80°C. Ten microlitres of this stored culture were inoculated into fresh Marine Broth (Difco Laboratories, Detroit, USA) and incubated overnight at 28°C under constant agitation

© 2006 The Authors
Journal compilation © 2006 Society for Applied Microbiology and Blackwell Publishing Ltd,

(100 min^{-1}). The grown cultures of *V. campbellii* were washed in autoclaved artificial seawater and added to the *Artemia* culture water at approximately 10^5 cfu ml^{-1}.

Aeromonas hydrophila LVS3 was used as feed for the nauplii (Defoirdt *et al*., 2005). The strain was grown overnight on Marine Agar (Difco Laboratories, Detroit, USA), suspended in sterile artificial seawater, autoclaved, and added to the *Artemia* culture water at approximately 10^7 cells ml^{-1}.

Comamonas testosteroni LMG19554 was grown for 24 h in Marine Broth (Difco Laboratories, Detroit, USA), washed in sterile artificial seawater and added to the *Artemia* culture water at approximately 10^7 cfu ml^{-1}.

Starvation experiment

After hatching, groups of 20 nauplii were transferred to new sterile 50 ml falcon tubes containing 20 ml of filtered and autoclaved artificial seawater. Autoclaved LVS3 bacteria (approximately 10^7 cells ml^{-1}) and/or PHB particles (1000 mg l^{-1}; average diameter 30 μm; Goodfellow, Huntingdon, UK) were added to the culture water. The falcon tubes were put back on the rotor and kept at 28°C. The survival of *Artemia* was scored daily during a period of 3 or 4 days for non-fed or fed nauplii respectively. All manipulations were performed under a laminar flow hood in order to maintain sterility of the nauplii. Each treatment was performed in triplicate. After 2 days of starvation, 10 nauplii of the LVS3-fed treatments were killed with absolute ethanol and stained with the fluorescent dye Nile Blue A (Ostle and Holt, 1982). The nauplii were examined with an Axioskop II microscope (Carl Zeiss, Jena, Germany) equipped with a Peltier-cooled single-chip digital camera (Orca IIlm; Hamamatsu, Massay, France) connected to a PC.

In vivo *challenge tests*

Challenge tests were performed as described by Defoirdt and colleagues (2005), with slight modifications. Briefly, after hatching, groups of 20 nauplii were transferred to new sterile 50 ml tubes that contained 20 ml of filtered and autoclaved artificial seawater. The tubes were inoculated with *V. campbellii* LMG21363 (except for the control, where no pathogen was added) and fed with LVS3. For the experiment with fatty acids, the fatty acids were dissolved in artificial seawater at different concentrations (25, 50 and 100 mM). The pH of the solutions was adjusted to 7 and the solutions were filtersterilized over a 0.22 μm filter (Millipore, Bedford, USA). For the experiment with PHB, PHB particles were added to the *Artemia* culture water at different concentrations (10, 100 and 1000 mg l^{-1}). After feeding and the addition of the appropriate chemical and/or bacteria, the falcon tubes were put back on the rotor and kept at 28°C. The survival of *Artemia* was scored 2 days after the addition of the pathogen. All manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and nauplii. Each treatment was performed in triplicate.

Statistics

Different treatments were compared by independent samples *t*-tests, using the SPSS software, version 12.0. Differences were considered significant if the *P*-value of the *t*-test was below 0.05.

Acknowledgements

This work was supported by the 'Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen' (IWT Grant No. 33205) and the 'Fonds voor Wetenschappelijk Onderzoek' (Project No. G0230.02N).

References

Alvarez, J.D., Austin, B., Alvarez, A.M., and Reyes, H. (1998) *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. *J Fish Dis* **21:** 313–316.

Amor, S.R., Rayment, T., and Sanders, J.K.M. (1991) Poly(3-hydroxybutyrate) *in vivo*: NMR and X-ray characterization of the elastomeric state. *Macromolecules* **24:** 4583–4588.

Anderson, A.J., and Dawes, E.A. (1990) Occurence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev* **54:** 450–472.

Bearson, S., Bearson, B., and Foster, J.W. (1997) Acid stress responses in enterobacteria. *FEMS Microbiol Lett* **147:** 173–180.

Bergheim, O. (1940) Toxicity of intestinal volatile fatty acids for yeast and *Esch. coli*. *J Infect Dis* **66:** 222–234.

Boon, N., Goris, J., De Vos, P., Verstraete, W., and Top, E.M. (2000) Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2*gfp*. *Appl Environ Microbiol* **66:** 2906–2913.

Cherrington, C.A., Hinton, M., Pearson, G.R., and Chopra, I. (1991) Short-chain organic acids at pH 5.0 kill *Escherichia-coli and Salmonella* spp without causing membrane perturbation. *J Appl Bacteriol* **70:** 161–165.

Defoirdt, T., Bossier, P., and Verstraete, W. (2005) The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*. *Environ Microbiol* **7:** 1239–1247.

Diggles, B.K., Moss, G.A., Carson, J., and Anderson, C.D. (2000) Luminous vibriosis in rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) phyllosoma larvae associated with infection by *Vibrio harveyi*. *Dis Aquat Org* **43:** 127–137.

Doi, Y. (1995) Microbial synthesis, physical properties and biodegradability of polyhydroxyalkanoates. *Macromol Symp* **98:** 585–599.

Durant, J.A., Lowry, V.K., Nisbet, D.J., Stanker, L.H., Corrier, D.E., and Ricke, S.C. (2000) Late logarithmic *Salmonella typhimurium* HEp–2 cell association and invasion response to short-chain fatty acid addition. *J Food Saf* **20:** 1–11.

Freier, T., Kunze, C., Nischan, C., Kramer, S., Sternberg, K., Saß, M., *et al.* (2002) *In vitro* and *in vivo* degradation studies for development of a biodegradable patch based on poly(3-hydroxybutyrate). *Biomaterials* **23:** 2649–2657.

Gogolewski, S., Jovanovic, M., Perren, S.M., Dillon, J.G.,

© 2006 The Authors
Journal compilation © 2006 Society for Applied Microbiology and Blackwell Publishing Ltd,

and Hughes, M.K. (1993) Tissue-response and *in vivo* degradation of selected polyhydroxyacids-polylactides (PLA), poly(3-hydroxybutyrate) (PHB), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/VA). *J Biomed Mater Res* **27:** 1135–1148.

Jendrossek, D. (1998) Microbial degradation of polyesters: a review on extracellular poly(hydroxyalkanoic acid) depolymerases. *Polym Degrad Stab* **59:** 317–325.

Jendrossek, D., and Handrick, R. (2002) Microbial degradation of polyhydroxyalkanoates. *Annu Rev Microbiol* **56:** 403–432.

Karunasagar, I., Pai, R., Malahti, G.R., and Karunasagar, I. (1994) Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture* **128:** 203–209.

Kashket, E.R. (1987) Bioenergetics of lactic acid bacteria: cytoplasmid pH and osmotolerance. *FEMS Microbiol Rev* **46:** 233–244.

Kasuya, K., Doi, Y., and Yao, T. (1994) Enzymatic degradation of poly[(R)-3-hydroxybutyrate] by *Comamonas testosteroni*-ATSU of soil bacterium. *Polym Degrad Stab* **45:** 379–386.

Lavilla-Pitogo, C.R., Leaño, E.M., and Paner, M.G. (1998) Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent vibrios in the rearing environment. *Aquaculture* **164:** 337–349.

McHan, F., and Shotts, E.B. (1993) Effects of short-chain fatty acids on the growth of *Salmonella typhimurium* in an *in vitro* system. *Avian Dis* **37:** 396–398.

Madison, L.L., and Huisman, G.W. (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol Mol Biol Rev* **63:** 21–53.

Marques, A., François, J.-M., Dhont, J., Bossier, P., and Sorgeloos, P. (2004) Influence of yeast quality on performance of gnotobiotically grown *Artemia*. *J Exp Mar Biol Ecol* **310:** 247–264.

Marques, A., Dinh, T., Ioakeimidis, C., Huys, G., Swings, J., Verstraete, W., *et al.* (2005) Effects of bacteria on *Artemia franciscana* cultured in different gnotobiotic environments. *Appl Environ Microbiol* **71:** 4307–4317.

Molina-Aja, A., Garcia-Gasca, A., Abreu-Grobois, A., Bolan-Mejia, C., Roque, A., and Gomez-Gil, B. (2002) Plasmid profiling and antibiotic resistance of *Vibrio* strains isolated from cultured penaeid shrimp. *FEMS Microbiol Lett* **213:** 7–12.

Mukai, K., Yamada, K., and Doi, Y. (1993) Enzymatic degradation of poly(hydroxyalkanoates) by a marine bacterium. *Polym Degrad Stab* **41:** 85–91.

Ostle, A.G., and Holt, J.G. (1982) Nile Blue A as a fluorescent stain for poly-β-hydroxybutyrate. *Appl Environ Microbiol* **44:** 238–241.

Pass, D.A., Dybadahl, R., and Manion, M.M. (1987) Investigations into the causes of mortality in the pearl oyster, *Pinctata maxima* (Jamson), in western Australia. *Aquaculture* **65:** 149–169.

Saito, T., Tomita, K., Juni, K., and Ooba, K. (1991) *In vivo* and *in vitro* degradation of poly(3-hydroxybutyrate) in rat. *Biomaterials* **12:** 309–312.

Shinomiya, M., Iwata, T., Kasuya, K.-I., and Doi, Y. (1997) Cloning of the gene for poly(3-hydroxybutyric acid) depolymerase of *Comamonas testosteroni* and functional analysis of its substrate-binding domain. *FEMS Microbiol Lett* **154:** 89–94.

Sorgeloos, P., Lavens, P., Léger, P., Tackaert, W., and Versichele, D. (1986) *Manual for the Culture and Use of Brine Shrimp* Artemia *in Aquaculture.* Gent, Belgium: Artemia Reference Center, Ghent.

Soto-Rodriguez, S.A., Roque, A., Lizarraga-Partida, M.L., Guerra-Flores, A.L., and Gomez-Gil, B. (2003) Virulence of luminous vibrios to *Artemia franciscana* nauplii. *Dis Aquat Org* **53:** 231–240.

Subasinghe, R.P., Bondad-Reantaso, M.G., and McGladdery, S.E. (2001) Aquaculture development, health and wealth. In *Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium.* Subasinghe, R.P., Bueno, P., Phillips, M.J., Hough, C., McGladdery, S.E., and Arthur, J.R. (eds). Bangkok: NACA, pp. 167–191.

Sun, C.Q., O'Connor, C.J., Turner, S.J., Lewis, G.D., Stanley, R.A., and Roberton, A.M. (1998) The effect of pH on the inhibition of bacterial growth by physiological concentrations of butyric acid: implications for neonates fed on suckled milk. *Chem-Biol Interact* **113:** 117–131.

Teo, J.W.P., Suwanto, A., and Laa Poh, C. (2000) Novel β-lactamase genes from two environmental isolates of *Vibrio harveyi*. *Antimicrob Agents Chemother* **44:** 1309–1314.

Teo, J.W.P., Tan, T.M.C., and Laa Poh, C. (2002) Genetic determinants of tetracycline resistance in *Vibrio harveyi*. *Antimicrob Agents Chemother* **46:** 1038–1045.

Van Immerseel, F., Cauwerts, K., Devriese, L.A., Haesebrouck, F., and Ducatelle, R. (2002) Feed additives to control *Salmonella* in poultry. *Worlds Poult Sci J* **58:** 501–513.

Van Immerseel, F., De Buck, J., Pasmans, F., Velge, P., Bottreau, E., Fievez, V., *et al.* (2003) Invasion of *Salmonella enteritidis* in avian intestinal epithelial cells *in vitro* is influenced by short-chain fatty acids. *Int J Food Microbiol* **85:** 237–248.

Verschuere, L., Rombaut, G., Huys, G., Dhont, J., Sorgeloos, P., and Verstraete, W. (1999) Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. *Appl Environ Microbiol* **65:** 2527–2533.

Weltzien, F.-A., Hemre, G.I., Evjemo, J.O., Olsen, Y., and Fyhn, H.J. (2000) β-Hydroxybutyrate in developing nauplii of brine shrimp (*Artemia franciscana* K.) under feeding and non-feeding conditions. *Comp Biochem Physiol B* **125:** 63–69.

Wolin, M.J. (1969) Volatile fatty acids and the inhibition of *Escherichia coli* growth by rumen fluid. *Appl Microbiol* **17:** 83–87.

Yu, J., Plackett, D., and Chen, L.X.L. (2005) Kinetics and mechanism of the monomeric products from abiotic hydrolysis of poly[(R)-3-hydroxybutyrate] under acidic and alkaline conditions. *Polym Degrad Stab* **89:** 289–299.

Zhang, X.-H., and Austin, B. (2000) Pathogenicity of *Vibrio harveyi* to salmonids. *J Fish Dis* **23:** 93–102.

© 2006 The Authors
Journal compilation © 2006 Society for Applied Microbiology and Blackwell Publishing Ltd,